USE OF DILUENT GARLIC EXTRACT SUPPLEMENTATION IN IMPROVING SEMEN QUALITY OF ROOSTERS

Hazim J. Al - Daraji
Department of Animal Resources, College of Agriculture, University of Baghdad

ABSTRACT

This study was designed to examine whether garlic extract (GE) supplementation could improve rooster sperm motility, viability, and morphology during *in vitro* storage for different periods (24, 48 or72 h). A total of 42 White Leghorn roosters, 22 – wk old randomly divided into 6 experimental pens (7 roosters each) were used in this study. The experimental groups were as follows : T1 = fresh, undiluted semen (control) ; T2 = semen diluted 1 : 1 with Lake diluent (LD) alone ; T3 = semen diluted 1 : 1 with GE alone, while T4, T5 and T6 represented semen samples diluted 1 : 1 with LD and supplemented with 1, 2 or 4 ml GE/100 ml of diluent, respectively.

Results denoted that semen incubation for 24, 48 or 72 h at the refrigerator temperature in the absence of GE (T1) was associated with a significant ($p < 0.05$) decrease in the mass activity and individual motility, and significant ($p < 0.05$) increase in the percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities. However, the inclusion of GE into the LD (T4, T5 and T6) significantly ($p < 0.05$) improved motility, viability and normality of spermatozoa acrosomes compared with control group (T1). Besides, T5 and T6 surpasses all other treatments in ameliorate the deterioration that found in the semen traits included in this study after *in vitro* storage for up to 72 h. In addition, T2 was superior to T3 as regards mass activity and individual motility, whereas there were no significant differences between these two treatments with relation to percentages of live spermatozoa and normal spermatozoa and acrosomes for semen samples stored for 24, 48 or 72 h.

In conclusion, supplementation of GE into avian semen diluents particularly at the doses of 2 and 4 ml GE/100 ml of diluent can be used as successful technique for depresses the detrimental effects of lipid peroxidation which could lead to sperms deterioration during *in vitro* storage for up to 72 h.

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استخدام مستخلص الثوم في مخففات السائل المنوي لتحسين نوعية السائل المنوي للديكة

حازم جبار الدراجي
قسم الثروة الحيوانية – كلية الزراعة – جامعة بغداد

المستخلص

تم تصميم هذه الدراسة لبحث امكانية اضافة مستخلص الثوم في مخففات السائل المنوي لتحسين حركة النطف وحيويتها ومظهرها خلال فترة خزنها في المختبر لمدد خزنية مختلفة (24 و 48 أو 72 ساعة). واستخدم فيها 42 ديك لكهورن ابيض عمر 22 اسبوع ، اذ تم توزيعها عشوائياً على ستة حظائر تجريبية يتكون كل منها من 6 ديكة. وتم تقسيم مجاميع التجربة كما يلي: المعاملة الاولى تمثل السائل المنوي الطازج غير المخفف (مجموعة السيطرة) والمعاملة الثانية تمثل السائل المنوي الذي تم تخفيفه بنسبة 1:1 بمخفف Lake (LD) من دون اية اضافة والمعاملة الثالثة تمثل السائل المنوي الذي تم تخفيفه بنسبة 1:1 بمستخلص الثوم لوحده ، في حين ان المعاملات 4 و 5 و 6 تمثل عينات السائل المنوي التي تم تخفيفها بنسبة 1:1 بمخفف LD مع اضافة مستخلص الثوم بتراكيز 1 و 2 و 4 مل/100 مل من المخفف على التوالي.

اشارت النتائج الى ان حفظ السائل المنوي بدرجة حرارة الثلاجة لمدة 24 أو 48 او 72 ساعة من دون اضافة مستخلص الثوم (T1) ادى الى انخفاض معنوي (أ > 0.05) في الحركة الجماعية والفردية للنطف والى ارتفاع معنوي في النسبة المئوية للنطف الميتة والمشوهة وتشوهات الاكروسومات. من ناحية ثانية ، فأن اضافة مستخلص الثوم الى مخفف LD (المعاملات 4 و 5 و 6) ادت الى تحسن معنوي في كل من الحركة والحيوية ومظهر النطف والاكروسومات بالمقارنة مع مجموعة السيطرة (T1). فضلاً على ذلك ، فأن المعاملتين T5 و T6 قد تفوقت على باقي معاملات مستخلص الثوم في تحسين التدهور الذي حصل في صفات السائل المنوي التي شملتها الدراسة الحالية بعد فترة خزن للسائل المنوي استمرت لغاية 72 ساعة. من ناحية اخرى ، فأن المعاملة T2 قد تفوقت على المعاملة T3 فيما يتعلق بالحركة الجماعية والفردية للنطف ، في حين لم تكن هنالك فروقات معنوية بين هاتين المعاملتين فيما يتعلق بالنسبه المئوية للنطف الحية والنطف ذات الشكل الطبيعي وسليمة الاكروسوم لعينات السائل المنوي التي تم خزنها لفترات 24 أو 48 أو 72 ساعة.

يستنتج من الدراسة الحالية ، ان اضافة مستخلص الثوم في مخففات السائل المنوي للديكة خصوصاً بالتراكيز 2 و 4 مل مستخلص ثوم/100 مل من المخفف يمكن ان تستخدم كتقنية ناجحة في الحد من التاثيرات الضارة لتأكسد الدهون والتي يمكن ان تؤدي الى تدهور النطف خلال فترة الخزن في الثلاجة لمدة قد تصل الى 72 ساعة.

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Introduction

Spermatozoa are unique in structure and chemical composition and are characterized by high proportions of polyunsaturated fatty acids (PUFAs) in the phospholipid fraction of their membranes. This characteristic composition confers to sperm plasma membrane the fluidity they require to undergo the membrane fusion events that characterize fertilization (30). However, high level of PUFAs increases the susceptibility of cells to free radical attack and lipid peroxidation. Therefore, antioxidant protection is a vital element in maintaining motility, viability, membrane integrity, and fertilizing ability (5). The major fatty acyl components of avian spermatozoa are arachidonic (20: 4 *n* – 6) and docosatetraenoic (22: 4 *n* – 6) acids (17). Thus avian spermatozoa are characterized by high amounts of C_{20-22} polyunsaturates of the *n* – 6 series, whereas long – chain fatty acids of the *n* – 3 series predominate in mammalian spermatozoa. However, it appears that the 20 : 4 *n* – 6 and 22 : 4 *n* – 6 present in avian spermatozoa performs an essential function in promoting optimal spermatozoa motility, viability and fertilizing capacity, as marked reductions in the amounts of these fatty acids in spermatozoa as a result of lipid peroxidation are associated with impaired sperm number, motility, viability and fertilizing ability (16).

Free radicals – atoms formed when oxygen interacts with certain molecules during normal bodily processes or from exposure to several environments. Garlic is one antioxidant that defends against free radicals damage, thereby preserving the body's healthy functioning (19). However, garlic antioxidants help scavenges free radicals – particles that can damage cell membranes, interact with genetic material and possibly contribute to the aging process as well as the development of a number of conditions including heart disease and cancer. Garlic antioxidants can neutralize free radicals and may reduce or even help prevent some of the damage they cause over time. In the study of Silagy and Haw (27), a total of 261 patents from 30 general practices were given either garlic powder or placebo. After a 12 week treatment period mean serum cholesterol levels dropped by 12 % in the garlic treated group and triglycerides and total lipid levels decreased by 17 % and 19 %, respectively compared to the placebo group.

Because garlic (*Allium sativum* L.) has an antioxidant activity, this study examines the potential role of garlic extract (GE) as an antioxidant in preserving roosters spermatozoa during *in vitro* storage for certain storage periods (24, 48 or 72 h).

Materials and Methods

Forty– two White Leghorn roosters, 22–wk old randomly divided into 6 experimental pens (7 each) were used in the experiment. Cocks fed a commercial layer ration *ad libitum*. Semen samples were collected from all roosters once a week for 10 consecutive weeks (22 – 32 weeks of age) by using the method of Lake and Stewart (18). Semen samples in each treatment pen were divided into 3 test tubes of 1 ml each to provide 3 replicates pooled samples per each treatment group. Therefore, there were 30 replicates for each treatment. The experimental groups were as follows : T1 = fresh, undiluted semen (control group) ; T2 = semen diluted 1:1 with LD alone ; T3 = semen diluted 1:1 with GE alone ; T4 = semen diluted with LD and supplemented with GE (1 ml/100 ml of diluent) ; T5 = semen diluted with LD and supplemented with GE (2 ml/100 ml of diluent) and T6 = semen diluted with LD and supplemented with GE (4 ml/100 ml of diluent). Experimental samples were stored at the refrigerator temperature (4 – 6 ^{0}C) for different storage times (24, 48 or 72 h.). An aliquot of semen from each group was evaluated at 24, 48 and 72 h of *in vitro* storage for mass activity, individual motility, and percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities.

Spermatozoa motility (movement in a forward) was estimated on a percentage basis by using the microscopic method of Sexton (26). The determination of percentage of dead spermatozoa was done by using a Fast green – stain – Eosin B stain – glutamate extender (6). Percentage of abnormal spermatozoa was evaluated by using a Gentian violet – eosin stain (1). As an alternative to evaluate the percentage of acrosomal abnormalities, staining procedure for fixed samples have been developed to distinguish which spermatozoa have retained or lost the acrosome (3). However, the extraction of garlic components was achieved according to the procedure that mentioned by Ohnishi and Ohnishi (21). Results were evaluated by analysis of variance. Differences between experimental group means were analyzed by Duncan's Multiple Range Test, using the ANOVA procedure in Statistical Analysis System (25).

Results and Discussion

The overall means of the treated groups regarding mass activity and individual motility at 0 h show that the differences between treated groups and the

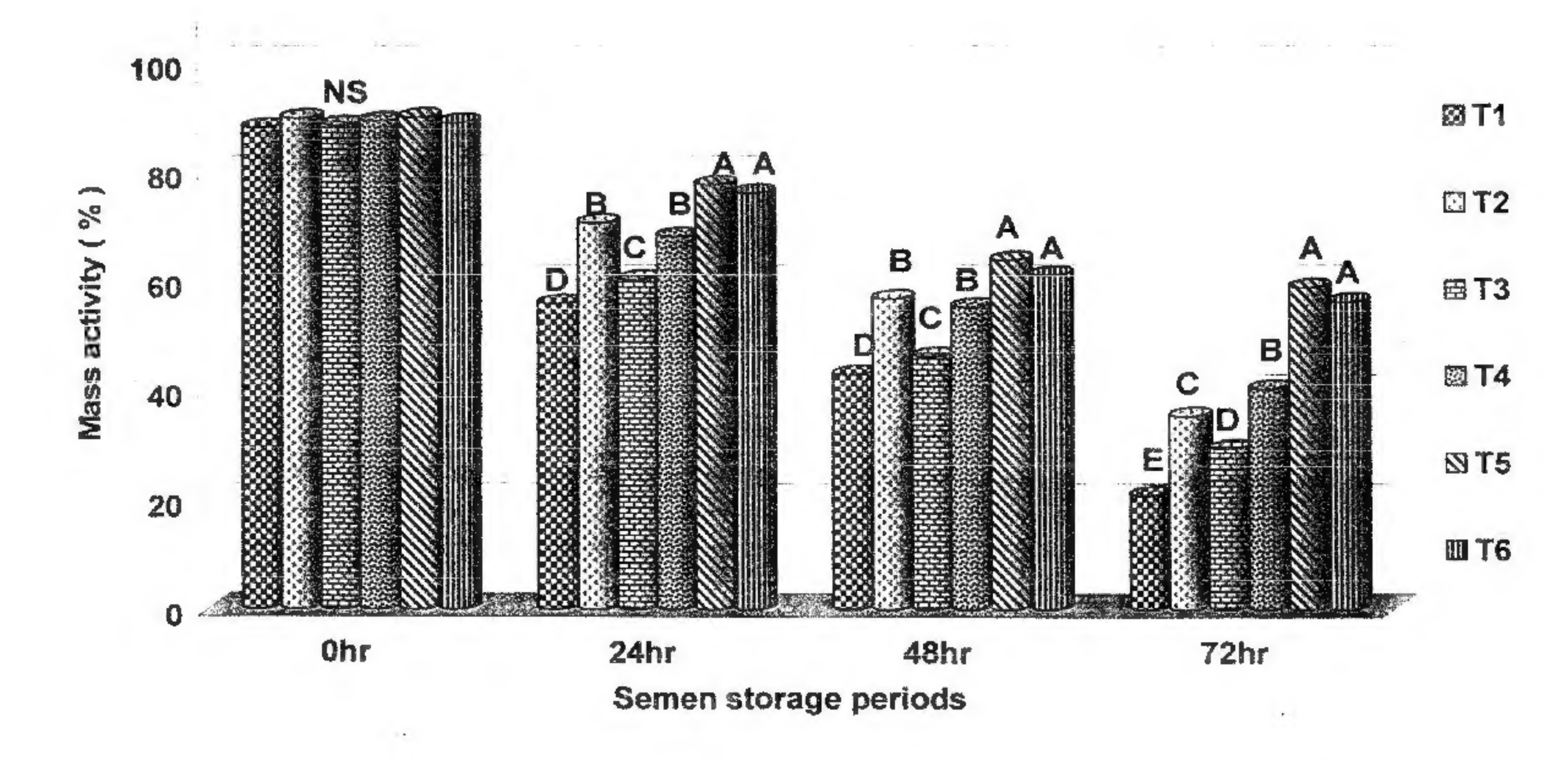
control were not statistically significant (Figures 1 and 2). However, in semen samples evaluated after 24, 48 or 72 h *in vitro* storage at the refrigerator temperature, diluent supplemented with GE (T4, T5 and T6) surpasses T1 and T3 groups as regards mass activity and individual motility. However, there were no significant differences between T2 and T4 groups with relation to these two traits when semen samples stored for 24 or 48 h, while T4 was superior to T2 group when samples evaluated after 72 h of storage. Besides, T5 and T6 surpass all other treatments in regard to mass activity and individual motility during all storage periods (Figures 1 and 2).

As shown in Figures 3, 4 and 5, there were no significant differences between T1, T2, T3 and T4 groups, and between T5 and T6 groups as regards percentages of dead and abnormal spermatozoa and acrosomal abnormalities for semen samples evaluated directly after collection (0 h). However, during this time of storage (0 h) T5 and T6 surpasses other treatments in relation to these three characteristics.In addition, supplementation of the diluent with GE (T4, T5 and T6) significantly improved the percentages of live spermatozoa and normal spermatozoa and acrosomes when semen samples *in vitro* stored for 24, 48 or 72 h. The most efficient levels were 2 ml GE/100 ml of diluent (T5) and 4 ml GE/100 ml of diluent (T6). On the other hand, there were no significant differences between T2 and T3 groups regarding these 3 characters (Figures 3, 4 and 5).

It is interestingly obvious from the results of this study that inclusion of GE into LD closely maintained activity, viability and normality of sperms and acrosomes. Our results are in accordance with the results of previous authors (2, 4, 5 and 12) who found that the addition of certain antioxidants (vitamins A, C or E) to the avian semen diluents had preserved motility, viability, morphology and fertilizing capacity of semen stored for different storage periods at 4 – 6 °C. Furthermore, the amelioration in semen characteristics noticed in the present experiment may was a result of GE antioxidants repressing or limiting the detrimental effects of lipid peroxidation during *in vitro* storage. Wishart (29) reported that lipid peroxidation is already initiated in fresh ejaculates and is able to develop during incubation even at low temperatures. Donoghue and Donoghue (11) pointed out that antioxidant activity in seminal plasma and sperm is not high enough to prevent lipid peroxide damage after extension and *in vitro* storage, and that supplemental antioxidants could improve semen shelf life.

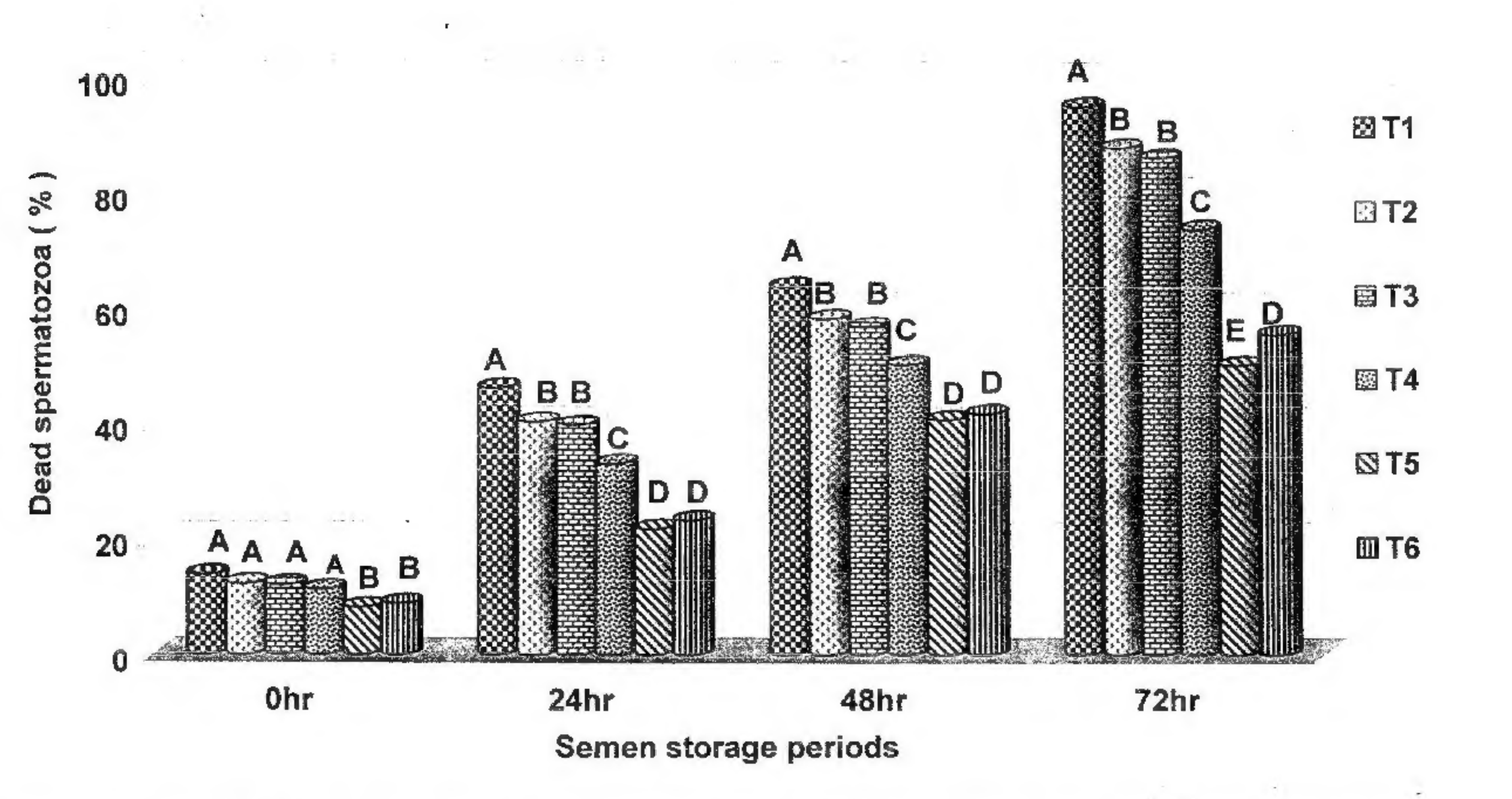
Extracts of fresh garlic contain antioxidant phytochemicals that prevent oxidant damage. These include unique water–soluble organosulfur compounds, lipid–soluble organosulfur components and flavonoids, notably allixin and selenium (21). A mounting body of research indicates that garlic act as a potent antioxidant, decreasing lipid peroxidation, increasing free radical scavenging and glutathione, lowering high cholesterol by interfering with its metabolism in the liver and lowering LDL (bad) cholesterol and triglyceride levels while raising the level of HDL (good) cholesterol (13 ; 23 ; 28). Ohnishi and Kojima (20) concluded that aged garlic extract has a strong antioxidant effect. However, GE exerts antioxidant action by scavenging reactive oxygen species (ROS), enhancing the cellular antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, and increasing glutathione in the cells (24). GE inhibits lipid peroxidation, reducing ischemic/reperfusion damage and inhibiting oxidative modification of LDL, thus protecting endothelial cells from the injury by the oxidized molecules (9). However, phytochemicals from plant – rich diets, including garlic, provide important additional protection against oxidant damage (10). The variety of antioxidant phytochemicals in GE, which protect against detriments-causing oxidative damage (14), may act in single and combined fashion (7). The antioxidative actions of GE and its components are determined by their ability to scavenge ROS and inhibit the formation of lipid peroxides. These effects are determined by measuring the decrease in ROS-induced chemiluminescence, inhibition of thiobarbituric acid reactive substances (lipid peroxides), and *in vitro* inhibition of the release of pentane, a product of oxidized lipids, in the breath of an animal exposed to oxidative stress (8 ; 15). On the other hand, Pizzorno et al. (22) reported that garlic has always been known as an aphrodisiac and from a medical point of view it can improve blood circulation significantly. Now appear that an enzyme called nitric oxide synthase is primarily responsible for the mechanism of erection. Studies have recently shown that garlic in certain forms can stimulate the production of nitric oxide synthase particularly in individuals who have low levels of this enzyme. Clearly folklore is now being proven correct.

Figure 1. Effect of supplementation of different levels of garlic extract into LD on mass activity of roosters semen stored for different periods .



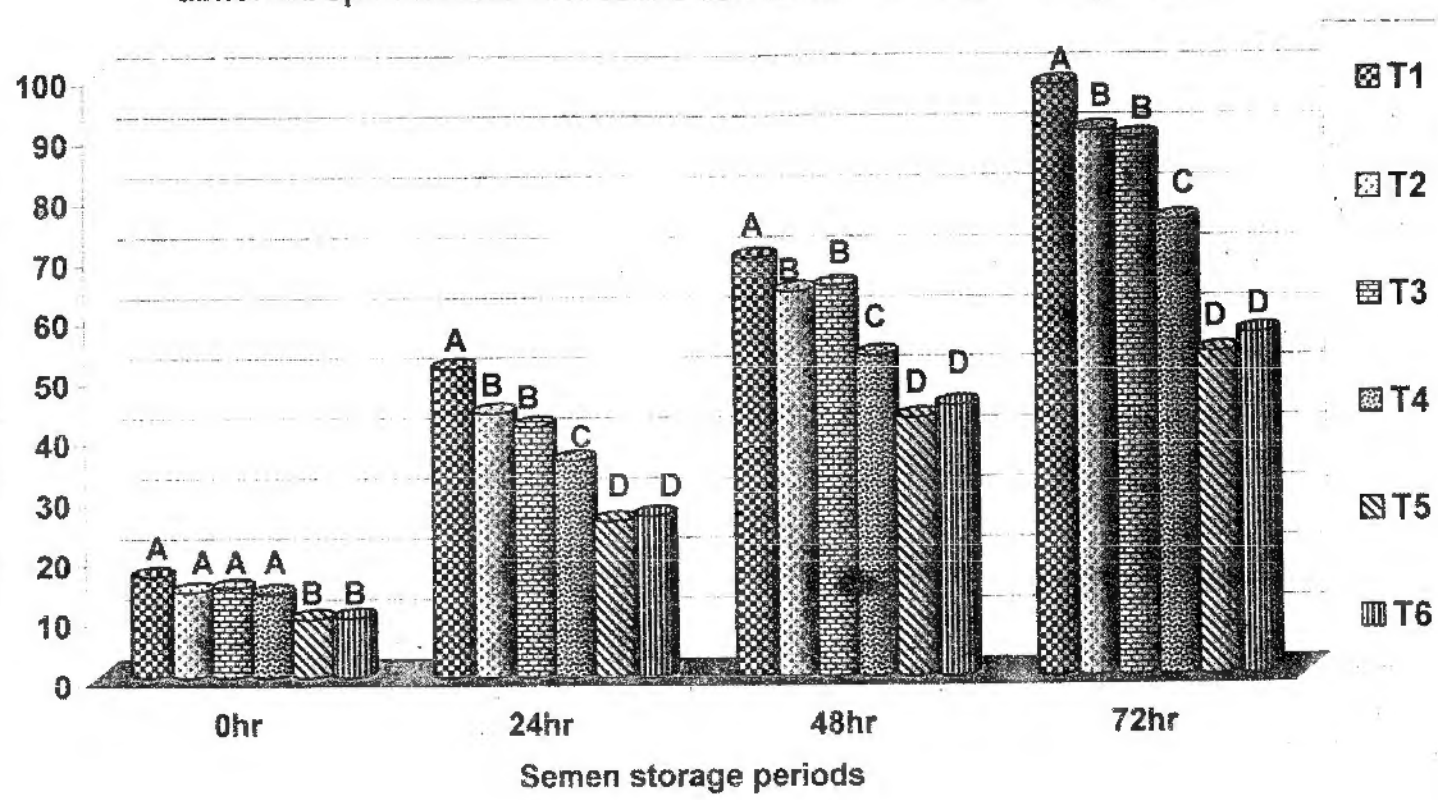
T1 = Fresh semen , T2 = LD , T3 = G , T4 = LD + G (1 ml / 100 ml) , T5 = LD + G (2 ml / 100 ml) and T6 = LD + G (4 ml / 100 ml) .
Bars with different superscripts differ significantly ($p < 0.05$) .

Figure 3. Effect of supplementation of different levels of garlic extract into LD on dead spermatozoa of roosters semen stored for different periods .



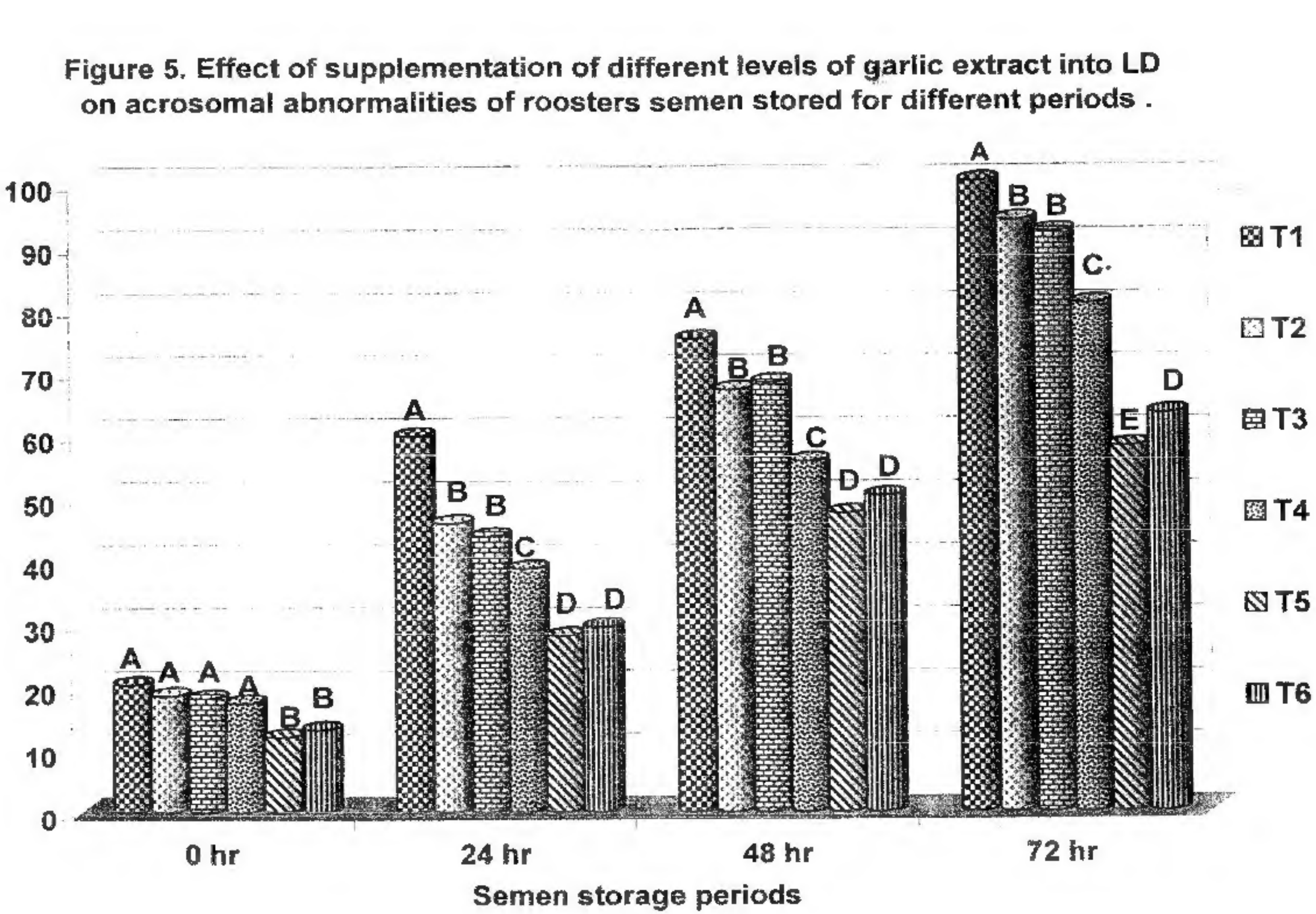
T1 = Fresh semen , T2 = LD , T3 = G , T4 = LD + G (1 ml / 100 ml) , T5 = LD + G (2 ml / 100 ml) and T6 = LD + G (4ml / 100 ml) .
Bars with different superscripts differ significantly ($p < 0.05$) .

Figure 4. Effect of supplementation of different levels of garlic extract into LD on abnormal spermatozoa of roosters semen stored for different periods .



T1 = Fresh semen , T2 = LD , T3 = G , T4 = LD + G (1 ml / 100 ml) , T5 = LD + G (2 ml / 100 ml) and T6 = LD + G (4 ml / 100 ml) .
Bars with different superscripts differ significantly ($p < 0.05$) .

Figure 5. Effect of supplementation of different levels of garlic extract into LD on acrosomal abnormalities of roosters semen stored for different periods .



T1 = Fresh semen , T2 = LD , T3 = G , T4 = LD + G (1 ml / 100 ml) , T5 = LD + G (2 ml / 100 ml) and T6 = LD + G (4 ml / 100 ml)
Bars with different superscripts differ significantly ($p < 0.05$) .

In the light of the results of the present study, it can be concluded that developing a defence system against lipid peroxide damages of practical importance to improve the extended liquid storage of roosters' semen. The present experiment demonstrated improved motility, membrane integrity, survival, and normality of spermatozoa and their acrosomes after cold storage for up to 72 h of roosters' sperms with garlic antioxidans that scavenge ROS in the sperm cell. Ultimately, the fertilizing ability of spermatozoa is most important and future studies will evaluate the effect of these antioxidants of garlic on this spermatozoa function.

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